WO9601273

Publication Title:

HELICOBACTER PYLORI ANTIGENIC PROTEIN PREPARATION AND IMMUNOASSAYS

Abstract:

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/205, G01N 33/569 — A1

(11) International Publication Number: **WO 96/01273**

(43) International Publication Date: 18 January 1996 (18.01.96)

(21) International Application Number: PCT/IE95/00037

(22) International Filing Date: 3 July 1995 (03.07.95)

(30) Priority Data:

940538	1 July 1994 (01.07.94)	IE
950249	6 April 1995 (06.04.95)	IE

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DE (Utility model), DK, DK (Utility model), EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(57) Abstract

A *Helicobacter pylori* protein preparation depleted of *H. pylori* antigens to which immunoreactivity is detected in *H. pylori* negative individuals and immunoassays using the protein preparation are described. The protein preparation is depleted of *H. pylori* antigens less than 30 kDa, especially 24 to 25 kDa and/or 18 to 19 kDa antigens.

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"*Helicobacter pylori* Antigenic Protein Preparation and Immunoassays"

Field of the Invention

This invention relates to an antigenic protein preparation obtained from *Helicobacter pylori*, and methods to use this protein preparation in diagnostic assays relating to *H. pylori*.

Background

Helicobacter pylori is a widely prevalent organism found on gastric biopsy in approximately 30% of the population less than 40 years old with increasing incidence thereafter. The organism is a causative agent of chronic gastritis in humans (e.g. Marshall & Warren 1984[1]; Blaser, 1990[2]). Epidemiological studies have shown that *H. pylori* is most commonly found in association with gastritis. Serological investigations have demonstrated that evidence of a current or prior infection can be found in 30 - 50% of a randomly chosen population of blood donors. No direct causal relationship has yet been conclusively proven for duodenal ulcer disease. However, the organism is found in 95% of patients with duodenal ulcer. Furthermore, eradication of the organism results in rapid ulcer healing (e.g. Rauws & Tytgat, 1990[3]). These data provide strong evidence that *H. pylori* is a dominant factor in the development of duodenal ulcer. Additional evidence for the pathogenic involvement of H. pylori in these conditions has been provided by studies with gnotobiotic piglets (Lambert et al., 1987[4]) and the fulfilment of Koch's postulates with human volunteers (Marshall et al., 1985[5]; Morris & Nicholson, 1987[6]).

In addition, there is now strong circumstantial evidence implicating *H. pylori* in the pathogenesis of gastric carcinoma (e.g. Jiang et al., 1987[7]; Lambert et al., 1986[8]; Crabtree et al., 1992[9]; 1993[10]; Forman et al.; 1990[11], 1991[12]; Nomura et al., 1991[13]; Parsonnet et al[14]).

Most recently, the Eurogast Study Group, led by Forman (1993[15]), demonstrated a significant relationship between *H. pylori* seropositivity and gastric cancer mortality and incidence. Indeed, there is now a convincing body of literature implying infection with *H. pylori* in a considerable proportion of upper gastrointestinal morbidity. A number of hypotheses have been suggested for the pathogenic mechanisms of *H. pylori* induced gastroduodenal disease, including the production of cytotoxins and mechanical disruption of the epithelium (e.g. Blaser, 1992[16]). Interestingly, however, many infected persons remain asymptomatic despite the persistent presence of the pathogen (Taylor & Blaser, 1991[17]).

Statements of Invention

According to the invention there is provided a *Helicobacter pylori* protein preparation depleted of *H. pylori* antigens to which immunoreactivity is detected in *H. pylori* negative individuals.

In a preferred embodiment of the invention, the immunoreactivity is antibody based.

In one particularly preferred embodiment of the invention the protein preparation is depleted of *H. pylori* antigens characterised by a molecular weight less than 30 kDa. The protein preparation may be depleted of *H. pylori* antigens characterised by a molecular weight of less than 29, preferably less than 28, or ideally less than 27 kDa.

In a preferred embodiment of the invention the protein preparation is depleted of antigen characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof.

In a preferred embodiment of the invention the protein preparation is depleted of antigens characterised by a molecular weight of approximately 18 to 19 kDa or derivative or fragment or precursor or mutant thereof.

Preferably the protein preparation is depleted of :-

(i) antigens characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof;

(ii) antigens characterised by a molecular weight of approximately 18 to 19 kDa or fragment or precursor or mutant thereof.

The 24 to 25 kDa antigen is further characterised in that it includes an N-terminal amino acid sequence shown in Sequence Id. No. 2 or portions thereof.

The 24 to 25 kDa antigen is further characterised in that it includes an internal amino acid terminal sequence shown in Sequence Id. No. 4 or portions thereof.

The 18 to 19 kDa antigen is further characterised in that it includes an N-terminal amino acid sequence shown in Sequence Id. No. 1 or portions thereof or preferably an N-terminal amino acid sequence listed in Sequence Id. No. 6 or portions thereof.

The 18 to 19 kDa antigen is further characterised in that it includes an internal amino acid terminal sequence shown in Sequence Id. No. 3 or portions thereof.

The antigen may be prepared as a glycine extract.

The invention also provides a method for detecting the presence of antibodies specific to *H. pylori* comprising contacting a test sample with an immunogenically effective amount of a *H. pylori* protein preparation of claims 1 to 13 to form, in the presence of said antibodies, detectable quantities of antigen/antibody complex, and then subjecting the complex to a detection means in order to detect the complex.

Preferably the test sample is selected from one of whole blood, serum, plasma, urine or a secretion such as a gastrointestinal secretion or saliva.

In a preferred embodiment of the invention the protein preparation is labelled or bound to a support, preferably a solid phase support.

Typically the solid support is a polystyrene plate or a nitrocellulose strip.

Preferably the detection means is a secondary antibody, conjugated with a reporter molecule, and which is specific for at least part of the *H. pylori* specific antibody found in the secretion.

The reporter molecule may be a fluorophore, a ligand such as a radio ligand or a gold ligand or an enzyme. The method may include the addition of a chromogen which is acted upon by the enzyme to produce a change in colour or optical density. Typically the enzyme is peroxidase and the chromogen is o-phenylenediamene (OPD).

The method may include the addition of a non-fluorescent substrate which is acted upon by the enzyme to produce a fluorescent substrate. Preferably the enzyme is ß-galactosidase and the non-fluorescent substrate is resosufin-ß-D-galactopyranoside.

The method may include the addition of a non-luminescent substrate which is acted upon by the enzyme to produce a luminescent substrate, typically the substrate is 3-(2^1-spiro-adamantane)-4-methoxy-(3^1phosphoryloxy)phenyl-1,2-dioxetane and the enzyme is alkaline phosphatase.

In one embodiment of the invention the sample is a human sample and the secondary antibody is rabbit anti-human immunoglobulin.

The invention further provides a test kit for detecting the presence of *H. pylori* in a test sample, the test kit comprising:

(a) a solid support having a protein preparation of the invention immobilised thereon; and

(b) detection means which in use detects whether *H. pylori* specific antibodies in the test sample binds to all or part of the protein preparation.

Preferably the test sample is selected from one of whole blood, serum, plasma, urine or a secretion such as a gastrointestinal secretion or saliva.

The support is preferably a solid phase support and may be a polystyrene plate or a nitrocellulose strip.

In one embodiment of the invention the detection means is a secondary antibody, conjugated with a reporter molecule, and which is specific for at least part of the *H. pylori* specific antibody found in the secretion. The reporter molecule may be a fluorophore, a ligand such as a radio ligand or a gold ligand or an enzyme.

The test kit may include a chromogen which, when acted upon by the enzyme, changes colour or optical density. Typically the enzyme is peroxidase and the chromogen is o-phenylenediamene.

In another case the kit includes a non-fluorescent substrate, which, when acted upon by the enzyme, becomes fluorescent.

Preferably the enzyme is ß-galactosidase and the non-fluorescent substrate is resosufin-ß-D-galacto-pyranoside.

In another case the kit includes the addition of a non-luminescent substrate which is acted upon by the enzyme to produce a luminescent substrate, typically the substrate is 3-(2^1-spiro-adamantane)-4-methoxy-

(3[1]phosphoryloxy)phenyl-1,2-dioxetane and the enzyme is alkaline phosphatase.

In a preferred embodiment of the invention the mammal is a human and the secondary antibody is rabbit anti-human immunoglobulin.

The invention also provides a method for detecting the presence of antibodies specific to *H. pylori* comprising the steps of :-

(a) contacting the protein preparation of the invention with a support suitable for use in agglutination assays;

(b) incubating said contactants of step (a) with a test sample to form, in the presence of *H. pylori* specific antibodies, agglutinated antigen-antibody complexes.

The support may comprise a plurality of latex beads or red blood cells.

The invention further provides a test kit for detecting the presence of *H. pylori* in a test sample, the test kit comprising an agglutination assay support having the protein preparation of the invention immobilised thereon. Preferably the support comprises glass or latex beads or the like. Alternatively, the support comprises red blood cells.

In this case the kit may also include means for incubating the agglutination assay support with a test sample.

The invention also provides the use of the protein preparation of the invention in an immunoassay.

Detailed Description

Fig. 1 : **Adult sera (CLO negative) screened for the presence of anti-*H. pylori* IgG antibodies.**

The figure shows a Western blot of *H. pylori* probed with serum obtained from CLO negative individuals. All sera were diluted 1:100 in PBS containing fat-free dried skimmed milk (5%, w/v). Proteins were transferred from SDS-PAGE gels to PVDF membrane. The antigen-antibody complexes were detected on washed membranes using an enhanced chemiluminescent detection system. Each track represents a different serum sample.

Fig. 2 : **Absorbed sera :** Sera from two individuals negative for *H. pylori* were absorbed with either whole *C. jejuni* (track A), *H. pylori* (track B), or *E. coli* (track C).

Fig. 3 : **Partial purification of 18 and 25 kDa proteins :** Both proteins were purified from whole *Helicobacter pylori* on the basis of molecular weight using preparative continuous-elution SDS-PAGE on a Model 491 Prep-Cell (Bio-Rad).

Fig. 4 : **Sera obtained from CLO negative children screened for the presence of anti-*H. pylori* IgG antibodies.** The figure shows a Western blot of *H. pylori* probed with serum obtained from CLO negative children. All sera were diluted 1:50 in PBS containing fat-free dried skimmed milk (5%, w/v). Each track represents a different serum sample.

Fig. 5 : **Antigens recognised on *C. jejuni* and *E. coli* by anti-*H. pylori* antiserum.** The figure shows a Western blot of *H. pylori* (track A), *C. jejuni* (track B) and *E. coli* (track C) probed with rabbit anti-*H. pylori* antiserum. Each bacterium (5 µg) was subjected to SDS-PAGE followed by immunoblotting.

Fig. 6 : Western blot of purified 25 kDa protein developed with serum from an individual negative for *H. pylori*. Purified 25 kDa protein was subjected to SDS-PAGE and Western blotting. The blot was probed with serum obtained from a subject uninfected with *H. pylori*.

Fig. 7 : Biotinylation of proteins located on the surface of *Helicobacter pylori*. Agar-grown *H. pylori* were harvested in phosphate buffered saline (pH 7.3) and washed twice in this buffer prior to biotinylation of surface exposed proteins. Bacteria (~2 mg ml^{-1}) were resuspended in PBS (1 ml) and prewarmed to 37°C. Thereafter, biotin-X-NHS (Sulfosuccinimidyl-6(biotinamido)-hexanoate; Calbiochem) was added to a final concentration of 1 mM and was prepared immediately before use. After mixing to 10 min at 37°C, the labelling reaction was terminated by the addition of 1.5 M Tris-Cl (pH 8) to a final concentration of 10 mM. The suspension was washed three times by centrifugation (10,000 *g*, 1 min) in ice-cold PBS. Examination of the bacteria by light microscopy after the labelling and washing procedures demonstrated that the cells were still intact and motile. Biotinylated *H. pylori* was subjected to analytical SDS-PAGE, followed by Western blotting, to identify the biotinylated proteins. The Western blots were developed with Extravidin-peroxidase (Sigma).

The present invention relates to improving the reliability of diagnostic immuno-assays for *Helicobacter pylori*. Previous studies (reference 3) have indicated a high level of false positive results in diagnostic immunoassays in which whole *H. pylori* protein has been utilised. Secondly, using specific antigens may limit the sensitivity of the immunoassay. The present

invention is based on observations using Western blot analysis of the antibody profile in individuals who are H pylori positive as defined by CLO test positivity and individuals who are H pylori negative as defined by CLO test negativity.

These studies identified two H pylori proteins of 19 and 25 kDa. Antibodies to these two proteins were detected in the majority of individuals who were CLO-negative on rapid urease testing. These antibodies were detected even in children who were CLO-negative. These proteins have been purified and N-terminal and internal and internal sequences identified as outlined in the Appendix. These proteins were the dominant conserved proteins to which immunoreactivity could be detected in H pylori negative individuals. These proteins could be depleted from the total protein preparation by preparative SDS-PAGE electrophoresis without resulting in significant depletion of other immunodominant antigens. As an example, this was most easily performed by simple elimination of proteins less than 30 kDa but could also be performed by elimination of proteins less than 29, 28 or 27 kDa.

The present invention relates to improving the reliability of serum, saliva or other mucous secretion-based immunoassays for H pylori. The present invention improves the specificity of immunoassays based on protein "mixtures" for H pylori by removing the 19 and 25 kDa proteins from the protein mixture. As an example, removal of these proteins by preparative SDS-PAGE analysts of all proteins less than 30 kDa from an antigenic preparation of *H. pylori* is cited. However, removal of such proteins might also be achieved by using affinity chromatography with antibody to these specific proteins for example.

An inherent constraint in the design of ELISA based detection systems is that of establishing a cut off point such that all samples below this threshold are considered negative. Clearly, many seropositive cases will remain undetected in this situation and a true

estimate of the incidences of prior contact with the organism will thereby be underestimated. Western blotting techniques were used to investigate antigen specificity of systemic responses to *H. pylori* in both healthy and *H. pylori*-infected individuals. The incidence of seropositivity in *H. pylori* negative individuals which has been shown is much greater than has previously been demonstrated. Furthermore, we have demonstrated that antibodies to a 25 kDa protein are detectable in the majority of *H. pylori* negative individuals. These were detected using a technique which we have modified called Enchanced Chemiluminescence. Enhanced Chemiluminescence on Western blot analysis reveals that the majority of uninfected individuals have antibodies which are specific for *H. pylori* and recognise antigens which are not present on other micro organisms. Of these antigens the most common one recognised is a 25 kDa protein which appears to be specific to *H. pylori*. A second protein was also identified at 18 kDa in a large subgroup of *H. pylori* negative individuals. Furthermore, our data indicates that depletion of these antigens improves the specificity of immuno-assay by removing antigens likely to result in false-positive analyses in *H. pylori* negative individuals.

An antigen component is present, for the purposes of this invention, if it is detectable by Western blot analysis. Conversely, it is absent if it is not detectable by this means.

Molecular weights of antigen components useful in the present invention are of necessity approximate figures, because of the limitations of current molecular weight determination procedures. The molecular weights specifically referred to have been obtained by a polyacrylamide gel electrophoresis (PAGE) system sold by BioRad under the trademarks PrepCell.

Those skilled in the art will be aware that slightly different results can be obtained in different hands or even on different occasions in the same hands, and so

the approximate molecular weight figures quoted in this specification should be read as + 5% or even ± 10%. For this reason, the 18 kDa antigen is sometimes referred to as the 18 to 19 kDa antigen.

The expression "antigen" is used in its broadest sense and includes whole *H. pylori* cells or homogeneous, near homogeneous or heterogeneous extracts from *H. pylori*, all of which are capable of binding to specific antibody in a serum or mucous secretion. Antigen components contemplated by the present invention include protein, polysaccharide or lipid or any combination thereof. Preferably, the antigen is protein, lipopolysaccharide or cell extract of *H. pylori* prepared, for example, by sonication, pressure disintegration, detergent extraction or fractionation.

In the method of the invention, antibody is detected in serum or bodily fluid secretion. By "bodily fluid secretion" is meant the secretion from epithelial cells such as those which line the canals, cavities and tracts that communicate with the external air, and in particular the nose, throat, respiratory tract, eyes, genital and urinary passages and the digestive system. In preferred embodiments, secretion is saliva or gastro intestinal secretion. Alternatively, this method can utilise any bodily fluid containing the antibody, such as blood, plasma, serum or urine.

The saliva or other mucous secretion may be assayed undiluted or diluted with an appropriate diluent (such as distilled water). With increasing sensitivities, dilution may be preferred (particularly when collection devices are used).

The antigen preparation will for convenience and preference be bound to a solid support. Suitable solid supports include a nitrocellulose membrane, glass or a polymer. The most commonly used polymers for this purpose are cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene, but the invention is not limited to them. The solid

supports may be in the form of strips, tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay.

Antigen components of *H. pylori* useful in this invention may be either covalently or non-covalently ("passively") bound to the solid surface. Suitable binding processes are well known in the art and generally consist of cross-linking, covalently binding or physically adsorbing the antigen to the solid support.

Infection is diagnosed by means of the present invention by detecting the formation of a complex between antibody in a serum or secretion sample and *H. pylori* antigens. In this particular case, the *H. pylori* antigen preparation is depleted of 18 to 19 and 24 to 25 kDa antigens. This is achieved by depletion of all antigens less than 30 kDa. Some form of detecting means is therefore necessary to identify the presence (or, if required, amount) of the antibody-antigen complex.

The detection means may be a second antibody, conjugated with a reporter molecule, and which is specific for at least part of the class of *H. pylori*-specific antibody found in the secretion.

ELISA

Immunoassays such as immunofluorescence assays (IFA), enzyme linked immunosorbent assays (ELISA) and immunoblotting can be readily adapted to accomplish the detection of the antigen. An ELISA method effective for the detection of the antigen can, for example, be as follows: (1) bind the antigen to a substrate; (2) contact the bound antigen with a fluid or tissue sample containing the antibody; (3) contact the above with a secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a colour reagent; (6) observe colour change. The above method can be readily modified to detect antibody as well as

antigen. A specific example of an ELISA of the present invention is provided in Example 5.

Micro-Agglutination Assay

A micro-agglutination test can also be used to detect the presence of the *H. pylori* antibodies in a subject. Briefly, latex beads (or red blood cells) are coated with the antigen and mixed with a sample from the subject, such that antibodies in the tissue or body fluids that are specifically reactive with the antigen crosslink with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible with the naked eye or by spectrophotometry. In a modification of the above test, antibodies specifically reactive with the antigen can be bound to the beads and antigen in the tissue or body fluid thereby detected.

Other Systems

In the diagnostic methods taught herein, the antigen can be bound to a substrate and contacted by a fluid sample such as serum, urine, saliva or gastric juice. This sample can be taken directly from the patient or in a partially purified form. In this manner, antibodies specific for the antigen (the primary antibody) with specifically react with the bound antigen. Therefore, a secondary antibody bound to, or labelled with, a detectable moiety can be added to enhance the detection of the primary antibody. Generally, the secondary antibody or other ligand which is reactive, either specifically with a different epitope of the antigen or nonspecifically with the ligand or reacted antibody, will be selected for its ability to react with multiple sites on the primary antibody. Thus, for example, several molecules of the secondary antibody can react with each primary antibody, making the primary antibody more detectable. This system using *H. pylori* antigen depleted of 18 and 25 kDa proteins can utilise any substrate for binding of the antigens preparation and

can use any detectable moiety to detect the primary antibody.

Detectable Moieties

The detectable moiety will allow visual detection of a precipitate or a colour change, visual detection by microscopy, or automated detected by spectrometry, radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by colour change) and immunogold. The detection methods and moieties used can be selected, for example, from the list above or other suitable examples by the standard criteria applied to such selections.

Conventionally, the antigen used is either a mixture of bacterial proteins or a purified bacterial protein to which there are circulated and secreted antibodies in the infected individual. Detection of such antibodies therefore denotes infection or exposure to the infectious agent. In the case of *Helicobacter pylori*, we have demonstrated that individuals uninfected with the organism have antibodies to either or both of two specific *H. pylori* protein species. Hence, removal of these species from the antigen mixture used for detection means removal of a significant source of error determining active infection with *Helicobacter* protein.

It is a further object of the present invention to increase the discriminatory power of ELISA testing for *H. pylori* by generating *H. pylori* protein preparations for use in ELISA tests from which the 18 kDa protein has been removed. Removal of a strongly immunogenic antigen to which antibodies are present in *H. pylori* negative individuals should increase the discriminatory capabilities of ELISA in identifying people with active infection.

It is also an object of the present invention to increase the discriminatory power of ELISA testing for by generating *H. pylori* protein preparations for use in ELISA tests from which the 18kDa protein and other antigens to which immunoreactivity is detected in *H. pylori* negative individuals- has been removed. Removal of a strongly immunogenic antigen to which antibodies are present in *H. pylori* negative individuals should increase the discriminatory capabilities of ELISA in identifying people with active infection.

It is an object of the current invention to provide other purified proteins of *H. pylori* to which constitutive antibodies are detected in *H. pylori* negative individuals.

It is also an object of the present patent to provide a *H. pylori* protein preparation from which proteins below 30 kDa have been removed on the basis for immunoassay for *H. pylori*.

We have developed a novel assay for detection of antibodies to *H. pylori*. This assay uses Western blotting and Enhanced Chemiluminescence (ECL). Using this assay we have demonstrated that approximately 75% of individuals who are negative for *H. pylori* by routine testing such as the rapid urease test have in fact got detectable antibodies to *H. pylori* (Fig. 1).

Furthermore, these antibodies are not absorbed by *C. jejuni* or by *E. Coli* suggesting that this is a specific antibody response (Fig. 2). Of particular note we have performed characterisation of the antigens recognised by these antibodies by molecular weight, using ECL Western blotting. Sera from n-infected individuals recognise a range of antigens on *H. pylori*. The most common antigen recognised is a 25 kDa protein which is recognised in over 70% of individuals who are negative for the organism on Rapid urease testing. Hence this suggests that the 25 kDa protein may be an immunodominant antigen which evokes a powerful immune response in individuals who are negative for the organism. A second protein was

identified at 18 kDa which elicited significant antibody responses in *H. pylori*-negative children.

METHOD SECTION

Methods used in the identification and partial purification of two novel antigens from *Helicobacter pylori*.

Methods

Western Blotting. Proteins from SDS-PAGE gels (30% T/ 2.67% C) were electroblotted (0.8 mA/cm^2 for 1 h) to PVDF membrane using a semi-dry blotting apparatus (LKB-Pharmacia). Primary antibodies (human serum; 1/50 - 1/100 dilution) were detected using a 1/5,000 dilution of anti-human IgG (horseradish peroxidase-conjugated) in combination with enhanced chemiluminescence (see below). Blots were washed in phosphate buffered saline (pH 7.5) containing fat-free dried skimmed milk (5%, w/v) and Tween-20 (0.05%, v/v). Blots were exposed to Kodak X-OMAT S film for 1-10 s. Exposed films were developed in Kodak LX-24 developer and fixed in Kodak dental X-ray fixer.

Enhanced Chemiluminescence (ECL)

The use of chemiluminescence to detect antibodies in Western blotting in preference to the conventional procedures of employing chromogenic substrates as detection reagents was adopted primarily because of the reporting gain in the sensitivity of detection (approximately 10-fold) over that found when chromogens are used. Oxidized luminol emits visible light and the intensity of this light emission is increased 1000-fold in the presence of chemical enhancers (e.g. iodophenol). The method is described below :

Substrate	Concentration/Amount
Luminol	1.2 mM (in 0.1 M-Tris (50ml), pH 8.8)
4-Iodophenol	0.4 mM (dissolved in DMSO before use)

Hydrogen Peroxide 17 µl of a 30% (v/v) solution

Blots were incubated in the above mixture for one minute and then exposed to X-ray film as described above.

Partial Purification of 18 and 25 kDa Proteins

Both proteins were partially purified from whole Helicobacter pylori on the basis of molecular weight (Fig. 2) using preparative continuous-elution sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Model 491 Prep-Cell (Bio-Rad). This method allows purification quantitatively of preparative amounts of proteins in a soluble form.

Purification Method

25 mg *H. pylori* were precipitated with ice-cold acetone, washed once in acetone and the precipitate then solubilised in 3.8 ml SDS-PAGE sample buffer (62 nM Tris, pH 6.8; glycerol (10%, v/v); SDS (2%, v/v); 2-mercapto-ethanol (5%, v/v); bromophenol blue (0.002%, v/v). Published electrophoretic procedures, with very minor modifications, were followed throughout sample preparation.

Loading: The protein mixture, in sample buffer, was loaded onto a 12.5% polyacrylamide tube gel (30% T/2.67% C). The dimensions of the tube gel were : 28 mm internal diameter; upper surface 3.6 cm^2; stacking gel 2 cm; resolving gel 10 cm.

Running Conditions: Electrophoresis was performed at 40 mA (constant current) overnight at room temperature. Fractions (1 ml) were collected at 0.1 ml/min. Samples of each fraction (5 µl) were subjected to analytical SDS-PAGE to assess the parity and antigenicity of each protein. Every fraction within the molecular mass region of interest was screened by both SDS-PAGE (to assess purity) and Western blotting (to assess antigenicity) in an attempt to isolate and characterise

the individual immunogenic proteins. The resolution of this technique is such that pure preparations of single proteins may be achieved once optimal electrophoretic conditions have been established. Preliminary optimization protocols entailed electro-phoresing mixtures of *H. pylori* proteins under conditions designed to favour high resolution of low molecular weight proteins. The final electrophoretic conditions used to achieve partial purification of the selected proteins are detailed in the Methods section. Using these exact conditions the 18 kDa proteins eluted between 11-14 ml and the 25 kDa protein eluted within 16-20 ml. The molecular weights of the proteins were determined by analytical SDS-PAGE using a range of low molecular weight marker proteins (range : 14.5 kDa - 66 kDa; code: Sigma SDS-7) and Western blotting confirmed that these proteins were the immunogens of interest.

Figure 1 shows Western blot analysis of antibody response to *H. pylori* in individual negative for *H. pylori* on Rapid urease testing. Western blotting was performed as previously described using an enhanced chemiluminescence detection system. Antibodies to a large range of *H. pylori* proteins were seen in individuals who are *H. pylori* negative on Rapid urease testing. The most common antigen to which an antibody was detected with the 25 kDa protein. Figure 3 shows a preparative SDS gel elution profile of the 25 kDa and 18 kDa proteins.

Detailed Description

Materials & Methods

Materials. All antibodies were obtained from Dako Ltd., High Wycome, Bucks., U.K. All other chemicals and solvents were obtained from either the Sigma Chemical Company Ltd., Poole, Dorset, United Kingdom or BDH Chemicals Ltd., Poole, Dorset, United Kingdom.

SDS-PAGE. Discontinuous SDS-PAGE was performed essentially as described by Laemmli (1970)[18]. A total of 5 mg of acetone-precipitated *H. pylori* protein were

located into each well. Gels were either stained with Coomassie Blue R-250 or processed for immunoblotting. Broad range molecular weight markers were purchased from Bio-Rad Laboratories, 3300 Regatta Blvd., Richmond, CA 94804. The molecular masses are expressed as kDa.

Western Blotting. Proteins from SDS-PAGE gels (30% T/ 2.67% C) were electroblotted (0.8 mA/cm^2 for 1 h) to PVDF membrane using a semi-dry blotting apparatus (LKB-Pharmacia), essentially as described by Towbin et al, (1979). Primary antibodies (human serum; 1/50 - 1/100 dilution) were detected using a 1/5,000 dilution of anti-human IgG (horseradish peroxidase-conjugated) in combination with enhanced chemiluminescence. Blots were washed in PBS containing fat-free dried skimmed milk (5%, w/v) and Tween-20 (0.05%, v/v). Blots were exposed to Kodak X-OMAT S film for 1-10 s. Exposed films were developed in Kodak LX-24 developer and fixed in Kodak dental X-ray fixer.

Sera. Serum samples were obtained from the Research Centre, Our Ladies Hospital for Sick Children, Crumlin, Dublin. All subjects were attended for medical conditions other than gastroenterological disorders. In addition, blood samples were obtained from a randomly selected cohort of children (Harcourt Street Childrens Hospital, Dublin) or from adults attending the gastenterology unit at St. James's Hospital, Dublin. All patients had a rapid urease (CLOtest) performed. Patients were defined as *H. pylori* positive or negative on the basis of positive or negative responses on rapid urease test.

Anti-*H. pylori* antiserum. Anti-*H. pylori* antiserum was a kind gift from Prof. B. Drumm and Dr. M. Clyne. The antiserum was raised in New Zealand white rabbits against whole *H. pylori* using conventional immunizing and boosting procedures.

Protein Measurements. Protein was measured by the method of Markwell et al. (1978)[19] with bovine serum albumin as the protein standard.

Absorption of sera. Antisera were absorbed with either *E. coli* or *C. jejuni* by incubating a suspension of the bacteria with patient sera for 2 h at room temperature with gentle mixing. The bacteria were removed from suspension by centrifugation (12,000 x g, 3 min).

Bacterial strains and growth conditions. The clinical isolates *H. pylori* used in this study were isolated from antral biopsies obtained from patients attending the gastroenterology clinic at St. James's Hospital, Dublin. *H. pylori* was grown under microaerophilic conditions for 4 days on 7% lysed horse blood agar at 37°C. Cells were harvested into ice-cold phosphate buffered saline (pH 7.5) containing PMSF (1 mM), EDTA (1 mM), and leupeptin (50 µg/ml). The cells were washed twice by centrifugation (10,000 x g, 5 min, 4°C) in this buffer before use. *C. jejuni* was a clinical isolate from stool in a patient with *C. jejuni* enteritis and was grown for two days exactly as described above with the exception that the incubation temperature was 42°C. The strain of *E. coli* used in this study is commercially available (Gibco) and was kindly provided by Dr. Ciaran Cronin, Dpt. Pharmacology, University College Dublin.

EXAMPLE 1

CLO negative adults

A cohort of 19 adult sera was screened for anti-*H. pylori* IgG antibodies. Each of these subjects was CLO negative, yet 83% had detectable antibodies (IgG) to *H. pylori* (Fig. 1). Taken together, these data suggest extensive prior contact with *H. pylori*. The most common antigen to which an antibody was detected was a 25 kDa species.

CLO negative children

The systemic humoral immune response (IgG) to *H. pylori* was studied in two groups of children also. None of these subjects had received any form of anti-*H. pylori* therapy. However, in almost all cases the children had

a specific antibody response to *H. pylori*. The first cohort studies consisted of twenty children (age range: 4 - 15 years), negative for *H. pylori* on CLO test. Of these, 75% had detectable IgG antibodies to *H. pylori* (Fig. 4).

The second cohort of children (n = 20) were asymptomatic and presented in hospital with conditions other than gastrointestinal disorders. Yet (note only 18/20 screened so far) 13/18 (72%) had detectable IgG antibodies to several *H. pylori* specific antigens. However, from the intensity of the response the data suggest that the antibody response is most likely due to prior contact with the bacterium, when compared to the considerably stronger response observed with *H. pylori* positive individuals.

EXAMPLE 2

Cross Reactivity with other Bacteria

As many bacteria share common antigenic determinants, we examined the extent of cross-reactivity between *H. pylori* and the closely related *C. jejuni*, in addition to *E. coli*, using two complimentary approaches. Firstly, the ability of the anti-*H. pylori* polyclonal antiserum to recognise antigens on both *C. jejuni* and *E. coli* was examined by Western blotting (Fig. 2).

Anti-*H. pylori* antiserum recognized a number of antigenic determinants on both *E. coli* and *C. jejuni*. Specifically, the antiserum recognises proteins of molecular mass 72, 50, 40, 36, and 25 kDa on *C. jejuni* and proteins of molecular mass 200, 116, 45, and 38 kDa on *E. coli* (Fig. 5). Of these, only 3 proteins (70, 25 kDa from *C. jejuni* and 200 kDa from *E. coli*) show pronounced cross-reactivity with anti-*H. pylori* antiserum. Therefore, the observed cross reactivity is clearly not extensive. Secondly, absorption experiments demonstrated that this cross reactive antigen recognition was of minor significance. Serum samples absorbed with clinical isolates of *H. pylori* and *C.*

jejuni in addition to a commercially available strain of *E. coli* demonstrated that seroreactivity could be eliminated by absorbing with *H. pylori* but not with *C. jejuni* or *E. coli* (Fig. 2). Figure 2 is a representative experiment. Absorption studies were performed on approximately half of the serum samples screened in this study with similar results to those shown. The 18 and 25 kDa proteins were also detected in *H. pylori* Reference Strains NTCC 11637 and 11638 in addition to all clinical strains tested.

Having partially purified the 26-26 kDa protein by preparation continuous-elution electrophoresis as shown in Fig. 3, we confirmed the antigenicity of the 24-26 kDa protein by probing a Western blot of purified 24-26 kDa protein with serum from an uninfected individual (Fig. 6). The example shown in Fig. 6 is a representative experiment where the blot was incubated with the serum from an *H. pylori* un-infected individual. Clearly, this serum sample contains antibodies that specifically recognise the 24-26 kDa protein and furthermore, the results of this experiment demonstrate that the antigen preparation is highly enriched for this protein and that no other immunogenic proteins are present in this preparation. We have obtained similar results with the 18-20 kDa protein.

EXAMPLE 3

Biotinylation of whole intact *Helicobacter pylori*

Agar-grown *H. pylori* were harvested in phosphate buffered saline (pH 7.3) and washed twice in this buffer prior to biotinylation of surface exposed proteins. Bacteria (~2 mg ml^{-1}) were resuspended in PBS (1 ml) and prewarmed to 37°C. Thereafter, biotin-X-NHS (Sulfosuccinimidyl-6(biotinamido)-hexanoate; Calbiochem) was added to a final concentration of 1mM and was prepared immediately before use. After mixing for 10 minutes at 37°C, the labelling reaction was terminated by the addition of 1.5 M Tris-Cl (pH 8) to a final concentration of 10 mM. The suspension was washed three

times by centrifugation (10,000 g, 1 min) in ice-cold PBS. Examination of the bacteria by light microscopy after the labelling and washing procedures demonstrated that the cells were still intact and motile.

Analysis of biotinylated proteins

Biotinylated *H. pylori* was subjected to both analytical and preparative SDS-PAGE, followed by Western blotting, to identify the biotinylated proteins. The Western blots were developed with Extravidin-peroxidase (Sigma). Extensive incorporation of the biotin ester into *H. pylori* proteins was observed (Fig. 7). Furthermore, it is clear from this figure that proteins in the 18-24 kDa region are biotinylated as are a number of other proteins (Table 1), indicating that these proteins are present on the surface of the bacterium.

Table 1

Biotinylated Protein	Apparent molecular weight
1	13,800
2	15,600
3	16,600
4*	17,700
5	20,500
6*	23,500
7	26,400

ELISA for detection of anti-*Helicobacter pylori* antibody using two different *H. pylori* antigen preparations.

Methods:

H. pylori strain NTCC 11637 were grown on 7% lysed horse blood agar under microaerophilic conditions at 37°C for 3 days (at least 20 plates). The bacteria were harvested in distilled water (1ml/plate). Then bacteria were then washed x 3 in distilled water by centrifugation at 3,000

rpm for 15 min at 4°C. The sediment was resuspended in distilled water (1:2 v/v). The cell suspension was then sonicated on ice using 6 x 15 s 100 Watt pulses, with 30 s cooling intervals in between, using a DAWE Soniprobe 7532A. The cell suspension was centrifuged at 2,500 rpm for 40 min. The supernatant was removed and the protein content estimated. A final protein concentration of 5 µg/ml is required for the test.

H. pylori antigens were purified on preparative SDS-polyacrylamide gel electrophoresis and protein having a molecular weight of less than 30 kDa were removed. A final protein concentration of 5 µg/ml is required for the test.

Both antigenic preparations were aliquoted and stored at -70°C until required.

H. pylori antigens were diluted with bicarbonate buffer and 100 µl of diluted antigens dispensed into each well of 96 well flat bottom plates respectively. The plates were incubated overnight in a humidified environment at 4°C.

Patient sera and control sera were diluted 1/400.

The plates were then washed three times using PBS-Tween 20 just before addition of serum. 100 µl of diluted serum are added into first and second rows and mixed gently twice in the second rows. Each test is titered, so doubling dilutions are performed from row B to row D.

The plates were incubated at 37°C for 60 min. Then the plates are washed three times using PBS-Tween 20.

The peroxidase-conjugated rabbit anti-human immunoglobulin was diluted with incubation buffer (1:2000). 200 µl of diluted conjugate was added into each well. The plates were incubated at 37°C for 30 min.

The plates were washed x 3 with by PBS-Tween 20. The substrate (o-phenylenediamene-OPD) was prepared fresh

prior to use and protected from light. 200 µl of diluted substrate was dispersed into each well. The plates were protected from light and left at room temperature for 15 min. The reaction was terminated by the addition of dilute H_2SO_4 (50 µl) and read at a wavelength of 492 nm.

An absorbance of 0.5 at 1/400 dilution was used as the cut-off point.

EXAMPLE 4 - Immunoassay

An example is provided whereby depletion of 25 and 19 kDa proteins, in this case by elimination of proteins less than 30 kDa resulted in improved specificity of *H. pylori* immunotesting. Positivity or negativity for *H. pylori* was defined in this case by CLO test results which have an approximately 90% correlation with bacterial culture performed on histological specimens. The use of protein preparations depleted of proteins <30 kDa permitted a significantly increased specificity for the assay without influencing sensitivity.

Table 1

Anti-*H. pylori* antibodies IgG levels in patients with "CLO" test positive or negative individuals. IgG levels were measured by *H. pylori* antigen from whole bacterium. Patients were designated as *H. pylori* positive on the basis of "CLO" test.

	10/42 HP+ve on "CLO" test	32/42 HP-ve on "CLO" test
Serology +ve	10/10 (100%)	12/32 (38%)
Serology -ve	0/10	20/32 (62%)

Table 2

Anti-*H. pylori* antibodies IgG levels in patients with "CLO" test positive or negative individuals. IgG levels

were measured by *H. pylori* antigen from protein greater than 30 kDa. Patients were designated as *H. pylori* positive on the basis of "CLO" test.

	10/42 HP+ve on "CLO" test	32/42 HP-ve on "CLO" test
Serology +ve	10/10(100%)	6/32(13%)
Serology -ve	0/10	28/32(87%)

It will be appreciated that while we have referred to a molecular mass of 24 to 25 kDa and 18 to 19 kDa the molecular mass may lie in the 24-26 kDa and 17-19 kDa range.

Partial sequencing of the two antigens from *Helicobacter pylori*

N-terminal sequence analysis

Purified 18 and 24 kDa proteins were electroblotted to PVDF and ProBlott, respectively, from 12.5% polyacrylamide gels. The proteins were located on the membranes by staining with 0.1% amido black (in 1% acetic acid, 40% methanol) for 15s followed by destaining in several changes of distilled deionized water. The membranes were air-dried thoroughly and submitted for sequence analysis using the Edman degradation procedure as described by Matsudaira (1989)[20].

The N-terminal amino acid sequence of the 25 and 18 kDa protein are given in Sequence Id No's 1 and 2 respectively.

Peptide Mapping

The N-chlorosuccinimide peptide mapping method of Lischwe and Ochs (1982)[21] was used with minor modifications. Bands of interest were located on SDS-

PAGE gels (12.5% T) by briefly staining the gel with 0.1% Coomassie Blue R250 (in 50% methanol, 10% acetic acid) and then excised with a scalpel blade. The protein present in the gel slices was digested with N-chlorosuccinimide (15 mM) in acetic acid/urea/water (1:1:1, v/w/v) for 30 min at 20°C. The treated gel slices were then washed with several changes of water and equilibrated with SDS-PAGE sample buffer exactly as described by Lischwe and Ochs. Finally, the gel slices were placed in the sample wells of a 15% polyacrylamide SDS-PAGE gel and electrophoresed. Following electrophoresis, the separated peptides were transferred to either PVDF or ProBlott by Western blotting. Peptides were visualized by staining the membrane with 0.1% amido black in acetic acid (1%) and methanol (40%). After extensive washing with water, the peptides were submitted for sequencing without any further modifications.

Mercaptoacetic acid (2 mM) was included in the upper electrode buffer during all SDS-PAGE electrophoretic procedures. This mobile thiol behaves as a free radical scavenger and thus prevents N-blocking.

Amino acid sequences for internal peptides from the 18 and 25 kDa protein are given in Sequence Id. No.'s 3 and 4 respectively.

Extraction of *Helicobacter pylori* chromosomal DNA

Chromosomal DNA was extracted as described (Silhavy et al., 1984. Experiments with gene fusions. C.S.H. publications).

Amplifying the sequence of the 18-19 kDa protein gene of using degenerate primers.

Degenerate DNA sequence was deduced from the amino acid sequences listed in Sequence Id. No.'s 2 and 3. Four degenerate primers were designed from these sequences, to allow for a two stage, nested, PCR reaction. *EagI* restriction enzyme sites were built into each primer, to

allow for subsequent cloning of the fragment. Where three or more bases were possible at any site, inosine was incorporated instead of all possible bases, except, where such sites were four bases or less from the primers 3' (3 prime) terminal, in which case all possible bases were included. Inosine was also avoided at positions immediately adjacent to the *Eag1* sites.

Degenerate primers for gene *p18*:

1. GAARA CGGCC GARAT IYTIA ARCAY YTICA RGC
2. TCYTC GGCCG TYTCY TCIGT NGCY
3. RATIY TCGGC CGYYI CARGC IGAYG C
4. ATYTC GGCCG TIGCY TTRTG NAC

Genomic DNA for the 18 - 19 kDa protein gene p18 was amplified as follows using the outer set of primers (primers 1 & 2): the samples were heated to 94 degrees C for 3 minutes to denature the DNA, followed by 35 cycles of 94 degrees C for 30 seconds, 56 degrees C for 40 seconds and 72 degrees C for 30 seconds. 100 pmol of each primer was used, in the presence of 2.5 mM $MgCl_2$ and 0.2 mM dNTPs, in a reaction volume of 50 ul. 1 ul of this reaction was used as the substrated for the 'nested' reaction. This reaction was the same as outlined for the above reaction, except that the inner primers (primers 3 & 4) were substituted for the external primers, and a concentration of 2.0 mM $MgCl_2$ was used. Electrophoresis of the products of the reaction resulted in a clearly visible band on a 2% agarose gel, estimated at approximately 120 bp in size (as judged by a molecular size ladder).

Sequencing the amplified DNA sequence.

The nested PCR fragment corresponding to the 18 - 19 kDa protein gene was cloned by digesting the fragment with *Eag1* and ligating this into the unique *Eag1* site in the Bluescript vector (Stratagene). E. coli cells were transformed (according to standard procedures) and plasmid DNA was harvested using the alkaline lysis method (Sambrook et al., 1989. Molecular cloning : A

laboratory manual 2nd. Ed., CSH publications) followed by an RNAase digestion step, phenol/chloroform extraction and precipitation using 2.5M ammonium acetate and 2 volumes of ethanol. Two independent isolates of plasmid DNA were sequenced using forward and reverse universal sequencing primers. The inserted DNA derived from the p18 gene was sequenced in the forward and reverse orientations. Sequencing was performed using an ABI automated sequencer and a Genpak PCR based fluorescent dideoxy chain terminator termini sequencing kit.

The sequence of bases between the terminal of the internal PCR primers is :

GATCGTGTTATTTATGAAAGTGCATAACTTCCATTGGAATGTGAAAGGCAC
CGATTTTTTCAAT

This sequence of bases translates into the amino acid sequence listed in Sequence Id. No. 5.

This sequence (Sequence Id. No. 5) overlaps with both the 18 kDa protein N-terminal amino acid sequence listed in Sequence Id. No. 2 and the 18 kDa protein internal amino acid sequence listed in Sequence No. 3, to give the enlarged N-terminal amino acid sequence listed in Sequence Id. No. 6.

Many variations on the specific embodiments described will be readily apparent and accordingly the invention is not limited to the embodiments hereinbefore described which may be varied in detail.

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APPENDIX

SEQUENCE LISTING

(1) GENERAL INFORMATION

(I) APPLICANT

(A) NAME : RICAN LIMITED
(B) STREET :1 STOKES PLACE,
(C) CITY :DUBLIN 2,
(D) COUNTRY :IRELAND
(E) POSTAL CODE :
(F) TELEPHONE :353-1-2881230
(G) TELEFAX : 353-1-2883439

(II) TITLE OF INVENTION : "*Helicobacter pylori* Antigenic Protein Preparation and Immunoassays"

(III) NUMBER OF SEQUENCES : 4

(IV)

(V) CURRENT APPLICATION DATA :6
APPLICATION NO. :

(2) INFORMATION FOR SEQUENCE ID. NO. : 1

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH : 20 AMINO ACIDS
(B) TYPE : AMINO ACID
(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

(XI) SEQUENCE DESCRIPTION : SEQ. ID. NO. 1

Met-Leu-Val-Thr-Lys-Leu-Ala-Pro-Asp-Phe-Lys-Ala-Pro-Ala-
5 10
Val-Leu-Gly-Asn-Asn-Glu
15

(3) INFORMATION FOR SEQUENCE ID. NO. 2. :

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH : 20 AMINO ACIDS
(B) TYPE : AMINO ACID
(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

(XI) SEQUENCE DESCRIPTION : SEQ. ID. NO. 2

Met-Lys-Thr-Phe-Glu-Ile-Leu-Lys-His-Leu-Gln-Ala-Asp-Ala-
5 10
Ile-Val-Leu-Phe-Met-Lys
15

NH2

(4) INFORMATION FOR SEQUENCE ID. NO. 3 :

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH : 20 AMINO ACIDS
(B) TYPE : AMINO ACID
(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

(XI) SEQUENCE DESCRIPTION : SEQ. ID. NO. 3

Asn-Val-Lys-Gly-Thr-Asp-Phe-Phe-Asn-Val-His-Lys-Ala-Thr-
5 10
Glu-Glu-Ile-Tyr-Glu-Glu
15 20

(5) INFORMATION FOR SEQUENCE ID. NO. : 4

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH : 4 AMINO ACIDS
(B) TYPE : AMINO ACID
(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

(XI) SEQUENCE DESCRIPTION : SEQ. ID. NO. 4

Lys-Asp-Thr-Pro

(6) INFORMATION FOR SEQUENCE ID. NO. 5:

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH - 21 AMINO ACIDS

(B) TYPE : AMINO ACID

(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

(XI) SEQUENCE DESCRIPTION : SEQ. ID. NO. 5

Ile-Val-Leu-Phe-Met-Lys-Val-His-Asn-Phe-His-Trp-Asn-Val-
5 10
Lys-Gly-Thr-Asp-Phe-Phe-Asn
15 20

(7) INFORMATION FOR SEQUENCE ID. NO. 6

(I) SEQUENCE CHARACTERISTICS

(A) LENGTH : 46 AMINO ACIDS

(B) TYPE : AMINO ACID

(C) TOPOLOGY : LINEAR

(II) MOLECULE TYPE : PROTEIN

(IV) ORIGINAL SOURCE :
(A) ORGANISM : HELICOBACTER PYLORI

Met-Lys-Thr-Phe-Glu-Ile-Leu-Lys-His-Leu-Gln-Ala-Asp-Ala-
5 10
Ile-Val-Leu-Phe-Met-Lys-Val-His-Asn-Phe-His-Trp-Asn-Val-
15 20 25
Lys-Gly-Thr-Asp-Phe-Phe-Asn-Val-His-Lys-Ala-Thr-Glu-Glu-
30 35 40
Ile-Tyr-Glu-Glu
45

CLAIMS

1. A *H. pylori* protein preparation depleted of *H. pylori* antigens to which immunoreactivity is detected in *H. pylori* negative individuals.

2. A *H. pylori* protein preparation as claimed in claim 1 wherein the immunoreactivity is antibody based.

3. A *H. pylori* protein preparation as claimed in claim 1 or 2 depleted of *H. pylori* antigens characterised by a molecular weight less than 30 kDa.

4. A *H. pylori* protein preparation as claimed in any of claims 1 to 3 depleted of *H. pylori* antigens characterised by a molecular weight less than 29 kDa.

5. A *H. pylori* protein preparation as claimed in any of claims 1 to 4 depleted of *H. pylori* antigens characterised by a molecular weight less than 28 kDa.

6. A *H. pylori* protein preparation as claimed in any of claims 1 to 5 depleted of *H. pylori* antigens characterised by a molecular weight less than 27 kDa.

7. A *H. pylori* protein preparation as claimed in any preceding claim depleted of antigens characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof.

8. A *H. pylori* protein preparation as claimed in any preceding claim depleted of antigens characterised by a molecular weight of approximately 18 to 19 kDa or derivative or fragment or precursor or mutant thereof.

9. A *H. pylori* protein preparation as claimed in any preceding claim depleted of :-

(i) antigens characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof;

(ii) antigens characterised by a molecular weight of approximately 18 to 19 kDa or fragment or precursor or mutant thereof.

10. A *H. pylori* protein preparation as claimed in any of claims 7 to 9 in which the 24 to 25 kDa antigen is further characterised in that it includes an N-terminal amino acid sequence shown in Sequence Id. No. 1 or portions thereof.

11. A *H. pylori* protein preparation as claimed in any of claims 7 to 10 in which the 25 kDa antigen is further characterised in that it includes an internal amino acid terminal sequence shown in Sequence Id. No. 4 or portions thereof.

12. A *H. pylori* protein preparation as claimed in claims 8 to 11 in which the 18 to 19 kDa antigen is further characterised in that it includes an N-terminal amino acid sequence shown in Sequence Id. No. 2 or portions thereof.

13. A *H. pylori* protein preparation as claimed in any of claims 8 to 12 in which the 18 to 19 kDa antigen is further characterised in that it includes an internal amino acid terminal sequence shown in Sequence Id. No. 3 or portions thereof.

14. A *H. pylori* protein preparation as claimed in claims 12 or 13 in which the 18 to 19 kDa antigen is further characterised in that it

includes an N-terminal amino acid sequence listed in Sequence Id. No. 6.

15. A *H. pylori* protein preparation as claimed in any preceding claim wherein the antigen is prepared as a glycine extract.

16. A method for detecting the presence of antibodies specific to *H. pylori* comprising contacting a test sample with an immunogenically effective amount of a *H. pylori* protein preparation of claims 1 to 14 to form, in the presence of said antibodies, detectable quantities of antigen/antibody complex, and then subjecting the complex to a detection means in order to detect the complex.

17. A method as claimed in claim 16 in which the test sample is selected from one of whole blood, serum, plasma, urine or a secretion such as a gastrointestinal secretion or saliva.

18. A method as claimed in claims 16 or 17 wherein the protein preparation is labelled or bound to a support.

19. A method as claimed in claim 18 wherein the support is a solid phase support.

20. A method as claimed in claim 19 wherein the support is a polystyrene plate.

21. A method as claimed in claim 19 wherein the support is a nitrocellulose strip.

22. A method as claimed in any of claims 16 to 21 wherein the detection means is a secondary antibody, conjugated with a reporter molecule, and which is specific for at least part of the *H. pylori* specific antibody.

23. A method as claimed in claim 22 wherein the reporter molecule is a fluorophore or a ligand such as a radio ligand or a gold ligand.

24. A method as claimed in claim 22 wherein the reporter molecule is an enzyme.

25. A method as claimed in claim 24 including the addition of a chromogen which is acted upon by the enzyme to produce a change in colour or optical density.

26. A method as claimed in claim 25 in which the enzyme is peroxidase and the chromogen is o-phenylenediamene (OPD).

27. A method as claimed in claim 25 including the addition of a non-fluorescent substrate which is acted upon by the enzyme to produce a fluorescent substrate.

28. A method as claimed in claim 27 in which the enzyme is ß-galactosidase and the non-fluorescent substrate is resosufin-ß-D-galactopyranoside.

29. A method as claimed in claim 24 including the addition of a non-luminescent substrate which is acted upon by the enzyme to produce a luminescent substrate, typically the substrate is 3-(2^1-spiro-adamantane)-4-methoxy-(3^1phosphoryloxy)phenyl-1,2-dioextane and the enzyme is alkaline phosphatase.

30. A method as claimed in any of claims 16 to 29 wherein the sample is a human sample and the secondary antibody is rabbit anti-human immunoglobulin.

31. A test kit for detecting the presence of *H. pylori* in a test sample, the test kit comprising:

(a) a support having a protein preparation of any of claims 1 to 15 immobilised thereon; and

(b) detection means which in use detects whether *H. pylori* specific antibodies in the test sample binds to all or part of the protein preparation.

32. A test kit as claimed in claim 31 in which the test sample is selected from one of whole blood, serum, plasma, urine or a secretion such as a gastrointestinal secretion or saliva.

33. A test kit as claimed in claim 31 or 32 wherein the support is a solid phase support.

34. A test kit as claimed in claim 33 in which the solid support is a polystyrene plate.

35. A test kit as claimed in claim 33 in which the solid support is a nitrocellulose strip.

36. A test kit as claimed in any of claims 31 to 35 wherein the detection means is a secondary antibody, conjugated with a reporter molecule, and which is specific for at least part of the *H. pylori* specific antibody.

37. A test kit as claimed in claim 36 wherein the reporter molecule is a fluorophore or a ligand such as a radio ligand or a gold ligand.

38. A test kit as claimed in claim 36 wherein the reporter molecule is an enzyme.

39. A test kit as claimed in claim 38 including a chromogen which, when acted upon by the enzyme, changes colour or optical density.

40. A test kit as claimed in claim 39 in which the enzyme is peroxidase and the chromogen is o-phenylenediamene.

41. A test kit as claimed in claim 38 including a non-fluorescent substrate, which, when acted upon by the enzyme, becomes fluorescent.

42. A test kit as claimed in claim 41 in which the enzyme is ß-galactosidase and the non-fluorescent substrate is resosufin-ß-D-galactopyranoside.

43. A test kit as claimed in claim 38 including the addition of a non-luminescent substrate which is acted upon by the enzyme to produce a luminescent substrate, typically the substrate is 3-(2^1-spiro-adamantane)-4-methoxy-(3^1phosphoryloxy)phenyl-1,2-dioextane and the enzyme is alkaline phosphatase.

44. A test kit as claimed in any of claims 31 to 43 in which the sample is a human sample and the secondary antibody is rabbit anti-human immunoglobulin.

45. A method for detecting the presence of antibodies specific to *H. pylori* comprising the steps of :-

(a) contacting the protein preparation of any of claims 1 to 15 with a support suitable for use in agglutination assays;

(b) incubating said contactants of step (a) with a test sample to form, in the presence of *H. pylori* specific antibodies, agglutinated antigen-antibody complexes.

46. A method as claimed in claim 45 in which the support comprises a plurality of latex beads.

47. A method as claimed in claim 46 in which the support comprises red blood cells.

48. A test kit for detecting the presence of *H. pylori* in a test sample, the test kit comprising an agglutination assay support having the protein preparation of claims 1 to 15 immobilised thereon.

49. A test kit as claimed in claim 47 in which the agglutination assay support comprises glass or latex beads or the like.

50. A test kit as claimed in claim 48 in which the agglutination assay support comprises red blood cells.

51. A test kit as claimed in any of claims 48 to 50 further including means for incubating the agglutination assay support with a test sample.

52. Use of the protein preparation of claims 1 to 15 in an immunoassay.

10^{-3} x apparent molecular weight

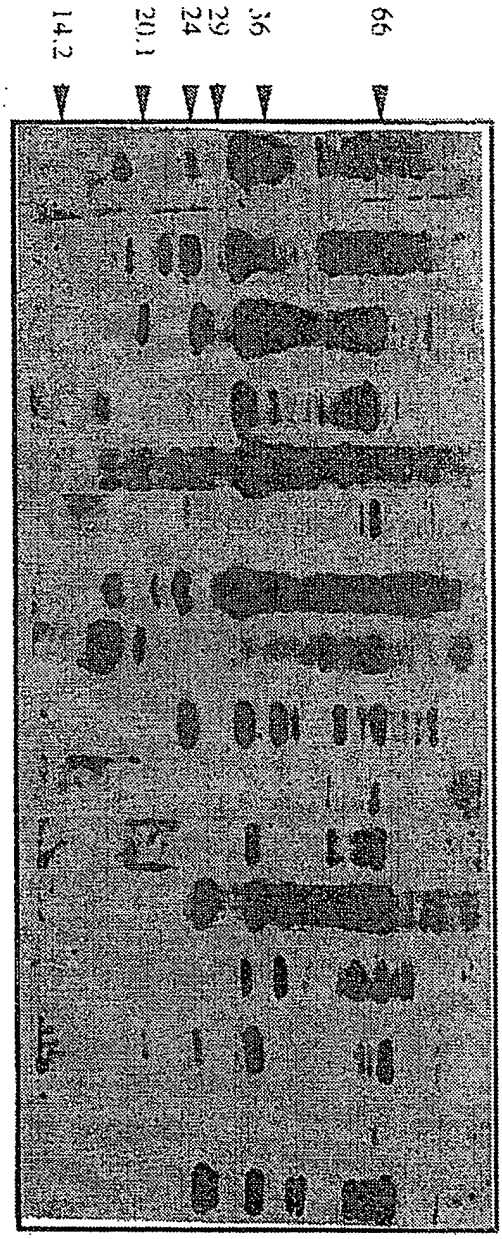
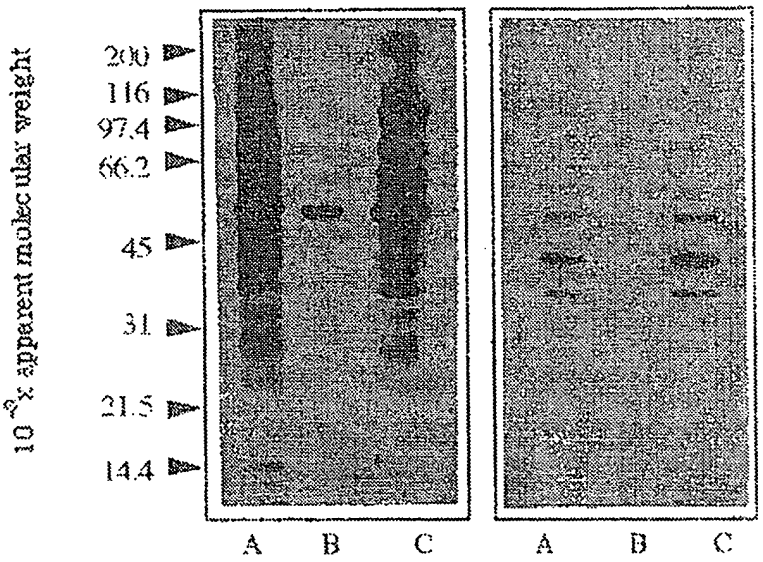


Fig 1


10^{-3} x apparent molecular weight
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97.4
66.2
45
31
21.5
14.4
A
B
C
A
B
C

Fig 2

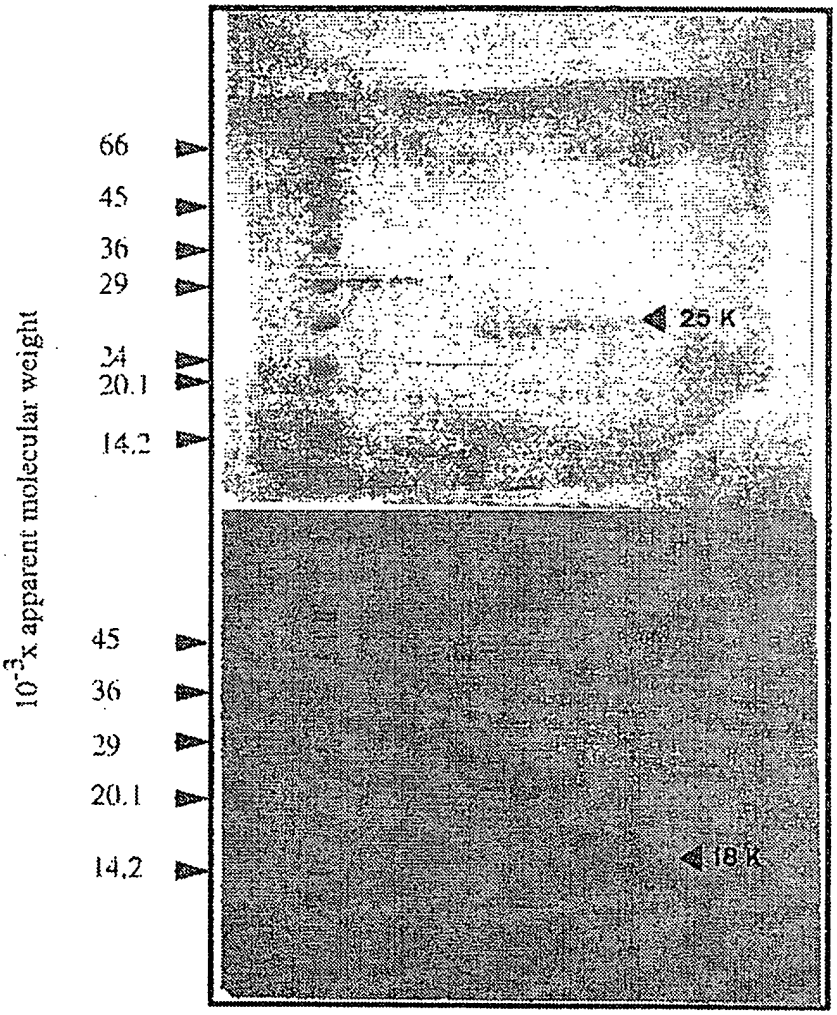
66
45
36
29
24
20.1
14.2
25 K
45
36
29
20.1
14.2
18 K
10^{-3}x apparent molecular weight

Fig 3

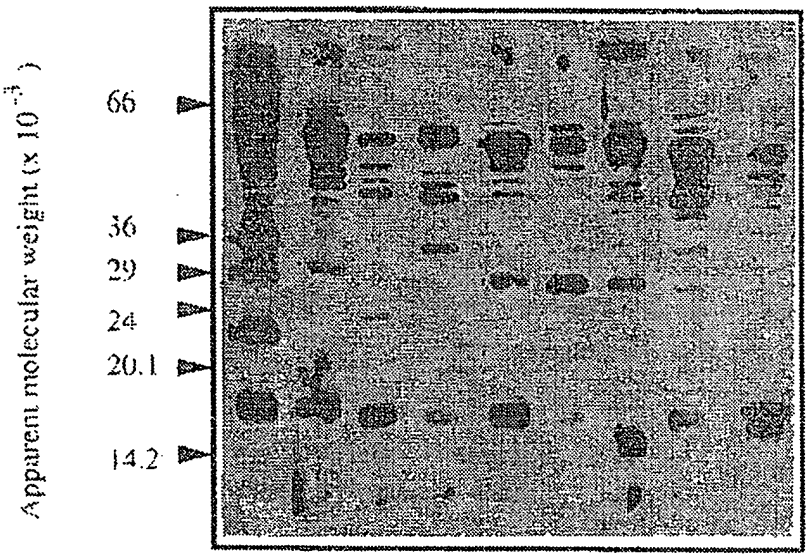

Fig 4

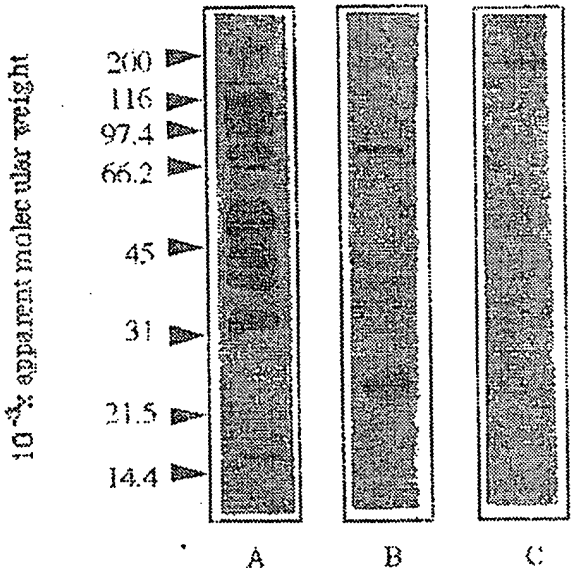
10⁻³ x apparent molecular weight
200
116
97.4
66.2
45
31
21.5
14.4
A
B
C

Fig 5

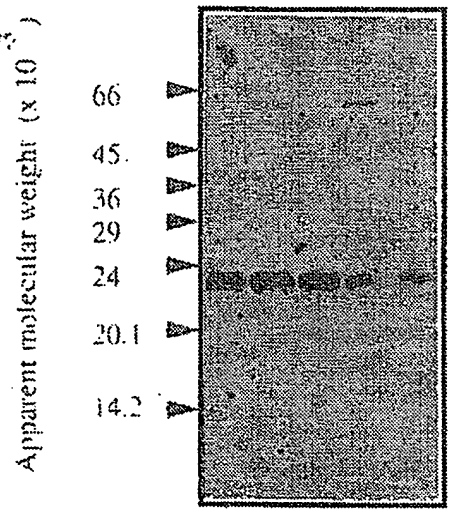

Fig 6

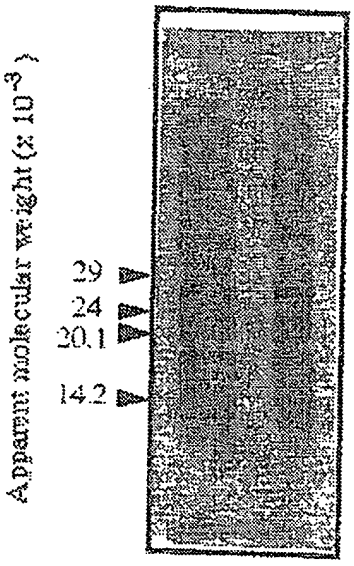

Fig 7

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/205 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol. 173, no. 2, January 1991 pages 505-513, O'TOOLE P.W. ET AL. 'Isolation and Biochemical and Molecular Analyses of a Species-Specific protein Antigen from the Gastric pathogen Helicobacter pylori' see the whole document ---	1-10,16
A	WO,A,91 09049 (RESEARCH EXPLOITATION LIMITED) 27 June 1991 see the whole document ---	1-52
A	FR,A,2 669 929 (QUIDEL CORPORATION) 5 June 1992 see the whole document -----	1-52

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 1995	21.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9109049	27-06-91	NONE		
FR-A-2669929	05-06-92	DE-A-	4139840	11-06-92
		IT-B-	1252164	05-06-95
		JP-A-	5264553	12-10-93